Supplementary Information

STAT5 promotes cell survival and restrains immunoglobulin κ gene recombination during B cell lineage commitment

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SUPPLEMENTARY METHODS

Mice

The *Stat5*$^{fl/fl}$ (ref. 1), *Stat5*$^{\Delta N/\Delta N}$ (ref. 2) and *Ebf1*$^{+/-}$ (ref. 3) mice were maintained on a mixed C57BL/6 x 129^{SV} background, whereas the *Bcl2l1*$^{fl/fl}$ (ref. 4), *Mcl1*$^{fl/fl}$ (ref. 5), *Rag1*$^{cre/cre}$ (ref. 6), *Il7r*$^{-/-}$ (ref. 7), *Rag2*$^{-/-}$ (ref. 8), *Tcfe2a*$^{fl/fl}$ (ref. 9), *Cd23-cre*[9] and *Aicda-cre*[9] and *Vav-bcl2* (ref. 10) mice were bred on the C57BL/6 genetic background. All experiments were performed with adult mice at the age of 4 to 6 weeks except for the immunization experiments, which were performed with 8-12-week-old mice. All animal experiments were carried out according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities.

Generation of *Ikzf1*^{Ebf1} transgene

The *Ikzf1* (Ikaros) BAC 054C2 (Research Genetics) was modified by homologous recombination[11] in *E. coli* using the following recombination cassette. A 1.3-kb NcoI-XhoI

fragment (3' region of upstream intron and first 15 bp of exon 2 of *Ikzf1*) and a 0.97-kb SpeI-EcoRI fragment (5' part of intron 2) were used as 5' and 3' homology regions to flank a 1860-kb mouse *Ebf1* cDNA fragment linked via the internal ribosomal entry sequence (IRES) to a *Gfp* gene and rabbit β-globin polyadenylation region. Injection of the modified $Ikzf1^{Ebf1}$ BAC DNA into pronuclei of C57BL/6xCBA F1 zygotes gave rise to the transgenic $Ikzf1^{Ebf1}$ line 10.

FACS analysis and sorting

FACS analyses were preformed with 4-6-week-old mice, unless otherwise stated. The following antibodies were used for flow cytometry: anti-B220 (RA3-6B2), CD3ε (145-2C11), CD4 (L3T4), CD5 (53-7.3), CD8a (53-6.7), CD11b/Mac1 (M1/70), CD11c (HL3), CD19 (1D3), CD21 (7G6), CD23 (B3B4), CD25/IL-2Rα (PC61), CD38 (90), CD43 (S7), CD44 (IM7), CD62L (MEL-14), CD90/Thy1.2 (53-2.1) CD117/c-Kit (2B8), CD127/IL-7Rα (A7R34), CD135/Flt3 (A2F10.1), DX5 (DX5), F4/80 (CI:A3-1), Gr1 (RB6-8C5), IgD (1.19), IgG1 (A85-1), IgM (M41.42), Ly6C (ER-MP20), Sca1/Ly6A (D7) and Ter119 (TER119). Bone marrow was isolated from the femur and tibia of the two hind legs. Single-cell suspensions of fetal liver, bone marrow, thymus and spleen were pre-incubated at 40 million cells per ml with CD16/CD32 Fc-block solution (PharMingen), and the different cell types were subsequently analyzed by flow cytometry or FACS-sorted as follows: CLPs (Lin^-IL-$7R\alpha^+Flt3^+Sca1^{lo}$c-$Kit^{lo}$), pre-pro-B (c-$Kit^+B220^+CD19^-IgM^-DX5^-Ly6C^-$), pro-B ($CD19^+$c-$Kit^+IgM^-$), pre-B ($CD19^+CD25^+IgM^-$), recirculating B ($CD19^+IgD^+$), marginal zone (MZ) B ($CD21^{hi}CD23^{lo}CD19^+B220^+$), follicular (FO) B ($CD21^{int}CD23^{hi}CD19^+B220^+$) and memory B cells ($CD4^-CD8a^-Gr1^-F4/80^-IgM^-[Lin^-]CD38^+IgG1^+NP^+CD19^+B220^+$). For CLP sorting, the following lineage marker antibodies were used: B220, CD3ε, CD4, CD5, CD8a, CD11a,

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CD11c, CD19, DX5, Gr1, Ly6C and Ter119. NP^+ memory B cells were stained with NP-TEG-biotin (Biosearch Technologies) followed by detection with PE-Cy5-streptavidin. The earliest $Thy1.2^+CD4^-CD8^-$ double-negative (DN) stages were defined as $CD44^+CD25^-$ (DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$ (DN3) and $CD44^-CD25^-$ (DN4) cells. All FACS data were analyzed by using a lymphocyte gate.

Intracellular staining

Thymocytes and splenocytes were incubated with CD16/CD32 Fc-block solution (PD PharMingen) and stained with a PE-Cy5-anti-CD4 antibody followed by permeabilization and intracellular staining with the PE-anti-Foxp3 antibody FJK-16s using the PE-Anti-Mouse/Rat Foxp3 Staining kit (eBiosciences).

Deletion analysis

DNA prepared from FACS-sorted cells was subjected to PCR genotyping using the following *Stat5* oligonucleotides: 5'-CCCCCTGAACCTGAAACATA-3' (primer 1), 5'-GGGCAGTCAGCAACGATAAT-3'(primer 2) and 5'-CAAATGAGTCAAGGCAGCAA-3' (primer 3). A 575-bp PCR product was amplified from the floxed (fl) *Stat5* allele with primer pair 1/2 and a 538-bp DNA fragment from the deleted (Δ) *Stat5* allele with the pair 2/3.

Antibodies

The following antibodies were used for Western blot analysis: rabbit polyclonal STAT5A and STAT5B (R&D Systems), Hsp90 (BD Biosciences) and Mcl-1 (Rockland) antibodies. Antibodies against the histone modifications H3K9Ac (07-352), H3K4me2 (07-030) and H3K4me3 (07-473) were purchased from Upstate Biotechnology for ChIP-chip analyses.

Histological analysis

Organs of autoimmune *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ and control *Stat5*$^{fl/fl}$ mice at the age of 4-12 weeks were fixed at 4ºC overnight with 4% formaldehyde in PBS followed by paraffin embedding, sectioning and staining with hematoxylin and eosin.

Immunization and ELISA measurements

Sheep red blood cells (SRBC) were washed in PBS and resuspended at 10^9 cells/ml followed by intraperitoneal injection of 100 µl into an adult mouse. To study the immune response to a specific antigen, *Cd23-cre Stat5*$^{fl/fl}$ and control *Stat5*$^{fl/fl}$ mice were intraperitoneally injected with 4-hydroxy-3-nitrophenylacetyl-conjugated keyhole limpet hemocyanin (NP-KLH; 100 µg in Alum Inject [Pierce]) at day 0 and with NP-KLH (10 µg in PBS) after 6 weeks. The serum titers of NP-specific antibodies were determined by enzyme-linked immuno-sorbant assay (ELISA) after 2, 4 and 8 weeks as described[9]. Briefly, the titers of different immunoglobulin isotypes were measured using plates, which were coated with NP_{30}-BSA or NP_3-BSA at 25 µg/ml to capture total or high-affinity NP-specific antibodies, respectively. Concentrations of NP-specific IgG1 were determined relative to that of a standard NP-binding IgG1 antibody (hybridoma SSX2.1).

RT-PCR analysis

Pro-B cells were FACS-sorted from the bone marrow of individual *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice or from the combined bone marrow of 7-11 adult *Vav-bcl2 Il7r*$^{-/-}$ mice. Pro-B cells were sorted into PBS containing 2% FCS prior to RNA preparation using the Absolutely RNA

Microprep kit (Strategene). cDNA was synthesized with random hexamers and superscript II reverse transcriptase (Invitrogen) followed by semiquantitative PCR analysis with primers (**Supplementary Table 1**) that amplified cDNA across exon-intron junctions. The PCR products were separated on agarose gels and visualized by ethidium bromide staining. Real-time PCR analysis was performed using the Light Cycler DNA Master SYBR Green 1 kit (Roche) and the qPCR primers listed in **Supplementary Table 1**.

V(D)J recombination analysis

Pro-B cells were FACS-sorted from the bone marrow of 4-6-week-old *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$**,** *Vav-bcl2 Il7r*$^{-/-}$ or control mice (**Supplementary Fig. 10**), and DNA was isolated by phenol/chloroform extraction and ethanol precipitation. PCR analyses of *Igh* and *Igk* rearrangements were performed with published primers exactly as described[12]. PCR cycle numbers were adjusted to be in the linear range, based on the analysis of serially diluted DNA. PCR products were separated on agarose gels, transferred to a porablot NYamp membrane and analyzed by Southern blotting using published oligonucleotide probes[12].

ChIP-chip analysis

Pro-B cells from the bone marrow of *Rag2*$^{-/-}$mice were expanded *in vitro* for 5 days on OP9 cells in the presence of IL-7, before they were subjected to chromatin immunoprecipitation (ChIP) as described in detail[13]. ChIP analysis was performed with purified antibodies (Upstate Biotechnology) recognizing the histone modifications H3K9ac (07-352), H3K4me2 (07-030) and H3K4me3 (07-473). Genomic DNA prepared from the chromatin-immunoprecipitated material and sheared input chromatin was subjected to T7-based linear amplification as described in detail[13] or to exponential amplification with the Whole Genome Amplification

(WGA) kit (Sigma). The amplified DNA was quality-controlled, quantified and sent to NimbleGen Systems for probe preparation and hybridization to a custom-made 50-mer oligonucleotide microarray, which contained the non-repetitive genomic sequences of the *Igh* locus among other selected genes at 100-bp resolution. The hybridization data were normalized based on the assumption that there should be on average no change between bound (immunoprecipitated) and input hybridization signals. We therefore corrected for different amounts of hybridized bound and input material, labeling efficiency and other experimental variations by calculating a robust average of the $\log_2$ (bound/input) intensities across the experimental probes using a one-step Tukey biweight function[14]. The resulting average was subtracted from all the $\log_2$ (bound/input) intensities effectively centering the $\log_2$ (bound/input) ratios at 0.

Statistical evaluation of the ChIP-chip data

The binding probability of each probe was determined using the single array error model[15]. A hidden Markov model[16] was used to combine the binding probabilities of neighboring probes (with an expected hybridization length of 3) into significantly enriched regions. Significantly enriched regions within 200 bp were combined into a contiguous block. Only regions with a false discovery rate above 900 were selected.

ChIP analysis of STAT5 binding

Wild-type pro-B cells were *in vitro* cultured in the presence of IL-7 (~2 ng/ml) and OP9 cells prior to IL-7 withdrawal for 4 h and subsequent restimulation with IL-7 (10 ng/ml) for 30 min. The cells were immediatey fixed in 1% formaldehyde for 10 min at room temperature followed by quenching with 0.125 M glycine for 5 min. All subsequent steps of the ChIP analysis were performed as previously described[13] except that the crosslinked chromatin was sheared to an

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average size of 200-300 bp using a Covaris sonicator and that the chromatin immunoprecipitation was performed with a mixture of STAT5A and STAT5B antibodies (R&D Systems). The sheared input material and precipitated DNA were analyzed by PCR amplification with primers shown in **Supplementary Table 2**.

LEGENDS TO SUPPLEMENTARY FIGURES

Supplementary Figure 1. Activity of the *Rag1*^{cre} allele in early lymphopoiesis.

(**a**) Cre reporter gene. The structure of the floxed *Tcfe2a* (E2A) allele with its exons is shown together with the insertion of six SV40 polyadenylation (pA) sites, a *Gfp* gene and a *neomycin* (*neo*) resistance gene downstream of the last *Tcfe2a* exon 19. *LoxP* and *frt* sites are indicated by red and yellow arrowheads, respectively. The *Gfp* gene contains the same 3' splice site (3'ss) as exon 17 and therefore gives rise to an E2A-GFP fusion protein upon Cre-mediated conversion of the *Tcfe2a*^{fl} to the *Tcfe2a*^{Gfp} allele. (**b**) Cre activity of the *Rag1*^{cre} allele in early lymphoid progenitors. GFP was already expressed in a subset (18%) of multipotent LSK cells (Lin$^-$IL-7Rα^-Sca1$^+$c-Kit^{hi}) in the bone marrow of *Rag1*$^{cre/+}$ *Tcfe2a*$^{fl/+}$ mice and was subsequently detected in all bone marrow pro-B cells (CD19$^+$c-Kit$^+$) and thymic DN1/2 pro-T cells (CD4$^-$CD8$^-$CD44$^+$c-Kit$^+$). These data demonstrate that the *Rag1*^{cre} allele initiates Cre-mediated deletion in multipotent hematopoietic progenitors and leads to complete gene deletion in all pro-B and pro-T cells. Consistent with these data, the *Stat5* allele was deleted in most FACS-sorted CLP cells of *Rag1*$^{cre/cre}$ *Stat5*$^{fl/fl}$ mice (**Supplementary Fig. 3c**).

Supplementary Figure 2. Autoimmune phenotype of *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice.

(**a**) PCR genotyping of the floxed (fl) and deleted (Δ) *Stat5* alleles in sorted DN thymocytes of the indicated genotypes. C, control *Stat5*$^{fl/\Delta}$ DNA. (**b**) Immunoblot analysis of total thymocytes. Threefold serial dilutions of whole cell extracts were analyzed with a mixture of STAT5A and STAT5B antibodies or a control Hsp90 antibody. (**c,d**) Flow cytometric analysis (**c**) and determination of absolute numbers (**d**) of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen of control *Stat5*$^{fl/fl}$ (black bar), *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ (grey bar) or *Vav-bcl2* *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (blue bar). n, number of 4-6-week-old mice analyzed. The expression of CD44 and CD62L is shown for gated $CD4^+$ T cells. Foxp3 expression was detected by intracellular staining. The percentages of cells are indicated for each quadrant or gate. Surprisingly, the restoration of normal numbers of $CD4^+CD25^+Foxp3^+$ Treg cells in *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice (**c,d**) still resulted in activated peripheral T cells ($CD44^+CD62L^-$) and autoimmunity, which may reflect the enhanced development of inflammatory Th17 cells in the absence of STAT5 (ref. 17). (**e**) Histological analysis of the liver of control *Stat5*$^{fl/fl}$ and autoimmune *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice. Areas of lymphocyte infiltrations are indicated by dark blue staining on tissue sections that were stained with eosin and hematoxylin.

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Supplementary Figure 3. Loss of B cell development upon *Stat5* inactivation in non-autoimmune mice.

(**a**) Flow cytometric analysis of fetal B lymphopoiesis. Pro-B (c-Kit$^+$CD19$^+$), pre-B (CD2$^+$CD19$^+$) and total B cells (B220$^+$CD19$^+$) were strongly reduced in the fetal liver of *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ (grey bar) compared to control *Stat5*$^{fl/fl}$ (black bar) embryos at E18.5. Absolute cell numbers of the different B cell types are shown to the right together with the number (n) of embryos analyzed. (**b**) Reduced bone marrow pro-B cell development in the absence of STAT5. Pro-B cells were strongly reduced in the bone marrow of *Rag1*$^{cre/cre}$ *Stat5*$^{fl/fl}$ compared to control *Stat5*$^{fl/fl}$ mice at the age of 4-6 weeks, whereas similar numbers of CLPs (Lin$^-$IL-7Rα^+Flt3$^+$Sca1^{lo}c-Kit^{lo}) were present in both genotypes. Importantly, the pro-B cell numbers were comparable in *Rag1*$^{cre/cre}$ and *Stat5*$^{fl/fl}$ mice, demonstrating that Cre expression from both *Rag1*^{cre} alleles did not lead to Cre-mediated toxicity. (**c**) Efficient *Stat5* deletion in CLPs of *Rag1*$^{cre/cre}$ *Stat5*$^{fl/fl}$ mice. CLPs were FACS-sorted as Lin$^-$IL-7Rα^+Flt3$^+$Sca1^{lo}c-Kit^{lo} cells to high purity and were then analyzed by PCR genotyping for the presence of floxed (fl) and deleted (Δ) *Stat5* alleles. C, genomic *Stat5*$^{fl/\Delta}$ DNA.

Supplementary Figure 4. Efficient *Stat5* deletion in pre-pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice

(**a**) FACS sorting of pre-pro-B cells, which were isolated as c-Kit$^+$B220$^+$CD19$^-$IgM$^-$DX5$^-$ Ly6C$^-$ progenitors from the bone marrow. The sorting is shown for *Vav-bcl2 Stat5*$^{fl/fl}$ mice. (**b**)

Efficient *Stat5* deletion in pre-pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice. Floxed (fl) and deleted (Δ) *Stat5* alleles were detected by PCR genotyping. C, genomic *Stat5*$^{fl/\Delta}$ DNA. (**c**) Semiquantitative RT-PCR analysis. The *Stat5a* mRNA was ~20-fold reduced in pre-pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice compared to control *Vav-bcl2 Stat5*$^{fl/fl}$ mice. Together, we analyzed three experimental mice (two in [**b**] and one in [**c**]), which showed efficient *Stat5* deletion in pre-pro-B cells.

Supplementary Figure 5. Loss of STAT5 protein in pre-pro-B and pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice

(**a**) Immunoblot analysis. Pro-B cells were sorted as c-Kit^{+}CD19^{+}CD25^{-}IgM^{-} cells from the bone marrow of wild-type (WT) or *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice prior to immunoblot analysis of whole cell extracts with a mixture of STAT5A and STAT5B antibodies or a control Hsp90 antibody. (**b**) Intracellular staining of STAT5 protein. Bone marrow cells of the indicated genotypes were stained with c-Kit, B220, CD19, DX5 and Ly6C antibodies, fixed with 2% formaldehyde, permeabilized with the Fix & Perm Cell Permeabilization kit (Caltag Laboratories) and stained with rabbit polyclonal STAT5A/B antibodies (R&D Systems) followed by detection with a FITC-labeled anti-rabbit antibody (Abcam). The staining of STAT5 expression is displayed for the gated c-Kit^{+}B220^{+}CD19^{-}DX5^{-}Ly6C^{-} pre-pro-B cells and c-Kit^{+}B220^{+}CD19^{+}DX5^{-}Ly6C^{-} pro-B cells. As pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice do not express STAT5 protein (**a**), the corresponding mean fluorescence of the intracellular staining (indicated by a red line) denotes the absence of STAT5 protein. As the same mean fluorescence is observed for pre-pro-B and pro-B cells of *Vav-bcl2 Rag1*$^{cre/+}$

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Stat5$^{fl/fl}$ mice, we conclude that the pre-pro-B cells of these mice also contain little or no STAT5 protein.

Supplementary Figure 6. Characterization of the *Ikzf1*^{Ebf1} transgene.

(**a**) *Ikzf1*^{Ebf1} transgene. *Ebf1* cDNA was inserted together with an *IRES-Gfp* gene into exon 2 of an *Ikzf1* (Ikaros) BAC. (**b-d**) GFP expression of the *Ikzf1*^{Ebf1} transgene in Lin^{-}Sca1^{hi}c-Kit^{hi} multipotent progenitors (LSK cells), c-Kit^{+}B220^{+} pro-B cells and different thymocyte subsets. Note that the ectopic expression of EBF1 disrupts T cell development in the thymus as previously published[18]. (**e**) Rescue of pro-B cell development in *Ebf1*$^{-/-}$ mice by ectopic EBF1 expression of the *Ikzf1*^{Ebf1} transgene. All rescued pro-B cells express GFP from the *Ikzf1*^{Ebf1} transgene. The percentage of c-Kit^{+}CD19^{+} pro-B cells is shown.

Supplementary Figure 7. EBF1 fails to rescue STAT5-deficient pro-B cell development. (**a**) Flow cytometric analysis of c-Kit^{+}CD19^{+} pro-B cells in the bone marrow of *Stat5*$^{fl/fl}$ (black bar), *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ (grey bar) and *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ *Ikzf1*^{Ebf1} mice (white bar) at the age of 4-6 weeks. Absolute pro-B cell numbers are shown to the right together with the number (n) of mice analyzed. (**b**) Absence of *Stat5a* mRNA in sorted pro-B cells from *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ *Ikzf1*^{Ebf1} mice, as shown by semiquantitative RT-PCR analysis of 5-fold serial cDNA dilutions. Note that *Ebf1* was only moderately overexpressed in pro-B cells of *Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ *Ikzf1*^{Ebf1} mice.

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Supplementary Figure 8. FACS sorting of memory B cells.

Mice were immunized with NP-KLH (100 μg in Alum) and, after 6 weeks, were boosted with NP-KLH (10 μg in PBS) followed by the isolation of memory B cells after additional two weeks. Memory B cells were purified from the spleen as $CD4^{-}CD8a^{-}Gr1^{-}F4/80^{-}IgM^{-}[Lin^{-}]CD38^{+}IgG1^{+}NP^{+}CD19^{+}B220^{+}$ cells[19] by FACS sorting using the gates indicated in red. The purity of the sorted memory B cells was determined by FACS reanalysis.

Supplementary Figure 9. Partial rescue of B cell development in *Il7r*$^{-/-}$ mice by transgenic Bcl-2 expression.

(**a**) Partial restoration of *Il7r*$^{-/-}$ pro-B cell development by transgenic Bcl-2 expression. Pro-B ($c\text{-}Kit^{+}CD19^{+}IgM^{-}$), pre-B ($CD25^{+}CD19^{+}IgM^{-}$) and total B cells ($B220^{+}CD19^{+}$) were drastically reduced in the bone marrow of *Il7r*$^{-/-}$ mice (grey bar) compared to control *Il7r*$^{+/-}$ mice (black bar) at the age of 4-6 weeks, whereas these B cell types were 5-10-fold increased in *Vav-bcl2 Il7r*$^{-/-}$ mice (blue bars). Absolute cell numbers of the different B cell types are shown to the right together with the number (n) of mice analyzed. (**b**) Size distribution of pro-B cells of the indicated genotypes, as determined by their forward scatter (FSC) profile. (**c**) Minimal rescue of splenic B cells compared to CD4 and CD8 T cells in *Vav-bcl2 Il7r*$^{-/-}$ mice.

Supplementary Figure 10. FACS sorting of STAT5- and IL-7Rα-deficient pro-B cells.

Pro-B cells were isolated from the bone marrow of a single *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mouse or the combined bone marrow of 7-11 *Vav-bcl2 Il7r*$^{-/-}$ mice at the age of 4-6 weeks. Pro-B cells were FACS-sorted as $c\text{-}Kit^{+}CD19^{+}CD25^{-}IgM^{-}$ pro-B cells by using the gates indicated in

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red, and the purity of the sorted pro-B cells was determined by FACS reanalysis. This sorting procedure yielded on average 100,000 STAT5-deficient pro-B cells per *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mouse and 5-10,000 IL-7Rα-deficient pro-B cells per *Vav-bcl2 Il7r*$^{-/-}$ mouse.

Supplementary Figure 11. Normal expression of *Tcfe2a*, *Ebf1* and *Pax5* in fetal liver pro-B cells of *Stat5*$^{-/-}$ embryos.

The expression of the indicated genes was analyzed by semiquantitative RT-PCR analysis using 5-fold serial dilutions of cDNA prepared from pro-B cells that were FACS-sorted from fetal livers of wild-type (+/+) and *Stat5*$^{-/-}$ (–/–) embryos at day 18.5. Only ~4.000 pro-B cells could be isolated from a *Stat5*$^{-/-}$ fetal liver due to a severe stem cell defect[20]. This analysis unequivocally demonstrates that *Tcfe2a* (E2A), *Ebf1* and *Pax5* are normally expressed in fetal liver pro-B cells of embryos lacking *Stat5* in the entire hematopoietic system.

Supplementary Figure 12. Lymphopoiesis in the absence of Bcl-xL and Mcl-1.

(**a**) B cell development in the absence of Bcl-xL and Mcl-1. Pro-B (c-Kit^{+}CD19^{+}IgM^{-}), pre-B (CD25^{+}CD19^{+}IgM^{-}), recirculating B (CD19^{+}IgD^{+}IgM^{lo}) and total B cells (B220^{+}CD19^{+}) were analyzed by flow cytometry in the bone marrow of control *Bcl2l1*$^{fl/fl}$ (black bar), *Rag1*$^{cre/+}$ *Bcl2l1*$^{fl/fl}$ (grey bar) and *Rag1*$^{cre/+}$ *Mcl1*$^{fl/fl}$ mice (denoted by 0). Absolute cell numbers of the different B cell types are shown to the right together with the number (n) of mice analyzed. Pro-B cells and all subsequent developmental stages were lost in the absence of Mcl-1, whereas the loss of Bcl-xL (encoded by *Bcl2l1*) strongly affected pre-B and later B cell development. (**b**) PCR genotyping of the floxed (fl) and deleted (Δ) *Bcl2l1* alleles in sorted pro-B cells of the

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indicated genotypes. (**c**) The absence of Bcl-xL led to the loss of DP and SP thymocytes, whereas Mcl-1 was already required for the survival of DN thymocytes. Absolute cell numbers are shown to the right for the different thymocyte subsets of control *Bcl2l1*$^{fl/fl}$ (black bar), *Rag1*$^{cre/+}$ *Bcl2l1*$^{fl/fl}$ (grey bar) and *Rag1*$^{cre/+}$ *Mcl1*$^{fl/fl}$ mice (white bar).

Supplementary Figure 13. No effect of Bcl2 expression on *Igh* recombination in pro-B cells. We have repeated the V(D)J recombination analysis shown in **Fig. 4a**, but this time used sorted c-Kit^{+}CD19^{+}CD25^{-}IgM^{-} pro-B cells from control *Vav-bcl2 Stat5*$^{fl/fl}$ mice instead of *Stat5*$^{+/+}$ mice for comparison with *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ pro-B cells. PCR analyses of D_H-J_H, V_H7183-DJ_H and V_HJ558-DJ_H rearrangements as well as of an *Igh* Cμ fragment (for normalization of input DNA) were performed as described in the legend of **Fig. 4a**. The presented data demonstrate that the frequency of D_H-J_H, V_H7183-DJ_H and V_HJ558-DJ_H recombination was similar in *Vav-bcl2 Stat5*$^{fl/fl}$ and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ pro-B cells and thus confirmed the result obtained with *Stat5*$^{+/+}$ and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ pro-B cells (**Fig. 4a**). We conclude therefore that transgenic Bcl-2 expression does not increase V(D)J recombination, although it may prolong the survival of pro-B cells.

Supplementary Figure 14. Normal V_HJ558-DJ_H recombination in *Stat5*$^{\Delta N/\Delta N}$ pro-B cells. (**a,b**) Genotyping of *Stat5*$^{\Delta N/\Delta N}$ mice. The N-terminally truncated (ΔN) STAT5A and STAT5B proteins were detected in thymocytes of *Stat5*$^{\Delta N/\Delta N}$ mice[2] by immunoblot analysis with a mixture of STAT5A and STAT5B antibodies. Hsp90 expression was analyzed to control for similar protein loading. (**c, d**) B cell development in 4-6-week-old *Stat5*$^{\Delta N/\Delta N}$ mice, as shown

by flow cytometric analysis (**d**) and determination of absolute cell numbers (**c**). Pro-B (c-Kit$^+$CD19$^+$IgM$^-$), pre-B (CD25$^+$CD19$^+$IgM$^-$) and total B (B220$^+$CD19$^+$) cells were reduced in the bone marrow of three *Stat5*$^{\Delta N/\Delta N}$ mice (genotyped in [**a**]) compared to three wild-type (WT) mice by a factor of 3, 6 and 5.5, respectively. (**e**) PCR analysis of distal V_H gene recombination. V_HJ558-DJ_H rearrangements occurred at a similar frequency in pro-B cells of wild-type and *Stat5*$^{\Delta N/\Delta N}$ mice at the age of 4-6 weeks (genotyped in [**a**]) as shown by semiquantitative PCR of 3-fold serially diluted DNA isolated from FACS-sorted pro-B cells. Input DNA was normalized by PCR amplification of a *Cd14* gene fragment. Numbers along the left margin indicate rearrangements to the J_H1, J_H2 and J_H3 gene segments. (**f**) RT-PCR analysis. V_HJ558 germline transcripts (GLT) as well as rearranged V_H7183-DJC_μ and V_HJ558-DJ_μ transcripts were similarly expressed in pro-B cells of wild-type and *Stat5*$^{\Delta N/\Delta N}$ mice (genotyped in [**b**]), as shown by semiquantitative RT-PCR analysis using 5-fold serial dilutions of cDNA prepared from FACS-sorted pro-B cells. In summary, these data demonstrate that the N-terminal truncation of STAT5A and STAT5B[2] facilitates pro-B cell development and has no effect on *Igh* recombination in our experiments, which contradicts the published data of Bertolino et al.[21]. We note that Bertolino et al.[21] isolated, by sequential MACS sorting, B220$^+$IgM$^-$ cells from the bone marrow of *Stat5*$^{\Delta N/\Delta N}$ mice for their D_H-J_H, V_H7183-DJ_H and V_HJ558-DJ_H rearrangement assays. These B220$^+$IgM$^-$ cells constitute, however, a heterogeneous mixture of pre-pro-B, pro-B and pre-B cells as well as plasmcytoid dendritic cells (pDCs) and nature killer cell precursors[22-24]. Moreover, it is well known that the pre-BCR selects the V_H gene repertoire for Igμ chains that are able to efficiently pair with surrogate light chains to form the pre-BCR[25]. Hence, Bertolino et al.[21] analyzed a mixture consisting of the primary V_H-DJ_H rearrangement repertoire of pro-B cells and the secondary, pre-BCR-selected

V_H-DJ_H rearrangement repertoire of pre-B cells. In contrast, we have investigated the primary V_H-DJ_H rearrangement repertoire in stringently sorted pro-B cells of control and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ mice. Hence, we conclude that Bertolino et al.[21] reached the wrong conclusion about the role of STAT5 in *Igh* recombination, as they analyzed a heterogeneous cell mixture of the hypomorphic *Stat5*$^{\Delta N/\Delta N}$ mutant mouse.

Supplementary Figure 15. Mapping of active histone modifications along the *Igh* locus in *Rag2*$^{-/-}$ pro-B cells.

(**a**) Structure of the *Igh* locus as described in the legend to **Fig. 5a**. (**b-f**) Mapping of active chromatin marks in the D_H-C_μ domain (**b**) and representative proximal (**c**) and distal (**d,e**) V_H gene regions of the *Igh* locus as well as at the 5' end of the *Ikzf1* (Ikaros) gene (**f**) in *Rag2*$^{-/-}$ pro-B cells. H3K9ac, H3K4me2 or H3K4me3 antibodies were used for ChIP-chip analysis[13] of *Rag2*$^{-/-}$pro-B cells, which were *in vitro* cultured for 5 days in the presence of IL-7 and OP9 cells. In contrast to the use of linearly amplified probes for generating the data shown in **Fig. 5**, the ChIP-precipitated DNA was this time amplified with the exponential whole-genome amplification method[26] prior to fluorescent labeling and co-hybridization with the input DNA probe onto a microarray containing the mouse *Igh* locus at 100-bp resolution (produced by NimbleGen Systems). The logarithmic ratio ($\log_2$) of the hybridization intensities between antibody-precipitated and input DNA (bound/input) is shown as a bar for each oligonucleotide on the microarray. The results shown are representative of two ChIP-chip experiments, which were performed with independently prepared samples of *Rag2*$^{-/-}$ pro-B cells. A scale bar is shown in kb. The mm8 sequence coordinates for the displayed regions of the *Igh* locus on

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chromosome 12 are 116,520,000-113,586,000 (**a**), 113,938,000-113,852,500 (**b**), 114,234,000-114,086,000 (**c**), 115,441,500-115,087,000 (**d**) and 115,859,000-115,780,000 (**e**).

Supplementary Figure 16. Absence of active chromatin at most V_H gene in WT pro-B cells. (**a**) Structure of the *Igh* locus as described in the legend to **Fig. 5a**. (**b-e**) Mapping of the active chromatin marks H3K9ac, H3K4me2 and H3K4me3 in the D_H-C_μ domain (**b**) and representative proximal (**c**) and distal (**d**) V_H gene regions of the *Igh* locus as well as at the 5' end of the *Ikzf1* (Ikaros) gene (**e**) in wild-type (WT, green) and *Rag2*$^{-/-}$ (black) pro-B cells. WT and *Rag2*$^{-/-}$pro-B cells were *in vitro* cultured for 5 days in the presence of IL-7 and OP9 cells, and differentiated CD25$^+$ pre-B cells (~5%) were removed by CD25 MACS sorting from the WT pro-B cell culture prior to ChIP-chip analysis based on whole-genome amplification of the precipitated DNA. The mm8 sequence coordinates for the displayed regions are indicated in the legend of **Supplementary Fig. 15**. WT pro-B cells have undergone D_H-J_H rearrangements and are in the process of V_H-DJ_H recombination at the *Igh* locus in contrast to the recombination-defective *Rag2*$^{-/-}$pro-B cells[27]. Nevertheless, the active chromatin marks H3K9ac, H3K4me2 and H3K4me3 could not be detected above background at V_H genes of the V_HQ52 (**c**), V_H7183 (**c**) and V_HJ558 (**d**) families in WT pro-B cells similar to *Rag2*$^{-/-}$pro-B cells. In contrast, all three active histone modifications were present in the Eμ enhancer region of the *Igh* locus (**b**) and at the promoter of the *Ikzf1* (Ikaros) gene (**e**). We conclude therefore that there is little or no active chromatin at most V_H genes of the *Igh* locus even in rearranging WT pro-B cells.

Supplementary Figure 17. Surface markers of Bcl-2-rescued STAT5-deficient pro-B cells. (**a,b**) Flow cytometric analysis. The cell surface phenotype of bone marrow pro-B (c-Kit$^+$CD19$^+$CD25$^-$IgM$^-$) and pre-B (CD19$^+$ CD25$^+$c-Kit$^-$IgM$^-$) cells was compared in control *Stat5*$^{fl/fl}$ (**a**) and *Vav-bcl2 Rag1*$^{cre/+}$ *Stat5*$^{fl/fl}$ (**b**) mice. The pro-B cells in mice of both genotypes expressed CD43 and c-Kit, but failed to express the 'pre-B cell' markers CD2 and MHC II in addition to CD25. In contrast, pre-B cells of both genotypes down-regulated CD43 expression and expressed CD2 and MHC II together with CD25. We conclude therefore that the Bcl-2-rescued STAT5-deficient pro-B cells express a cell surface phenotype that is characteristic of pro-B cells despite the observed increase of *Igk* and *Igl* rearrangements (**Fig. 7**).

Supplementary Figure 18. Direct binding of STAT5 to the intronic enhancer of the *Igk* locus in pro-B cells.

(**a**) Schematic diagram of the proximal region of the *Igk* locus. MAR, matrix attachment region. (**b**) Binding of STAT5 to the intronic iEκ enhancer. IL-7 was withdrawn for 4 h from *in vitro* cultured wild-type pro-B cells followed by restimulation with IL-7 (10 ng/ml) for 30 min prior to formaldehyde fixation and ChIP analysis with STAT5A and STAT5B antibodies. The immunoprecipitated (IP) DNA and 5-fold serial dilutions of the input chromatin (1/100) were analyzed by PCR for enrichment of known regulatory sequences of the *Igk* locus. Unspecific DNA precipitated in absence of STAT5 antibodies (but presence of protein A sepharose beads) or the absence of DNA template (H_2O) served as specificity controls for the PCR amplification. One representative result of three ChIP experiments is shown. (**c**) Sequence of the 3' region of the iEκ enhancer. Two known E-boxes (κE1 and κE2) and the NF-κB recognition sequence are shown together with a putative STAT5-

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binding site and the location of the ChIP PCR primers (arrows). Nucleotides matching the STAT5 consensus recognition sequence TTCnnnGAA[28] are indicated in boldface letters.

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